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A Study of Structure-Activity Relationships Among Drugs Which Affect Nicotine-Sensitive Physiological Mechanisms

Since the last report was submitted we have devoted our efforts to developing a sensitive and reliable bioassay procedure to permit measuring the acetylcholine concentration of biological and other fluids which contain nicotine, tetraethylammonium ion, hexamethonium ion and other agents which normally interfere with the assay of acetylcholine itself. The assay we have developed has been used to measure the influence of nicotine, tetraethylammonium, and hexamethonium on the acetylcholine content of incubation mixtures containing guinea pig ileal segments as generators of acetylcholine.

In our last progress report we detailed the experimental results which led us to believe that the impairment of ganglionic transmission produced by nicotine (and tetraethylammonium ions and morphine) may be the result of drug-induced impairment of release of acetylcholine from pre-synaptic nerve terminals, while the impairment of transmission produced by hexamethonium ions is probably the result of an action of the drug to prevent access of acetylcholine to post-synaptic receptors. Testing our hypothesis experimentally will ultimately necessitate measuring the acetylcholine output from ganglia in the presence and absence of impairment of transmission produced by ganglioplegic agents.

METHODS:

All determinations of acetylcholine were performed using the isolated rat ileum preparation; a total of more than eighty experiments have been performed.

After a rat of either sex of about 150 gms. weight has been killed by a blow on the head, a section of ileum is removed and washed inside and out with glucose-free Tyrode's solution. A segment of ileum about 2.5 cm. long is suspended in glucose containing Tyrode's solution in a bath of 5 ml. capacity; contractions of the ileum are recorded kymographically with an ink-writing isotonic lever which provides 6-fold magnification of the contraction. Tension on the muscle varies from 0.25 to 2.0 grams, in different experiments, but is constant during any one experiment.

The muscle bath containing the ileal segment is suspended in a water bath, the temperature of which is controlled to within 0.5° C. of 37° C. The water bath serves to preheat Tyrode's solution admitted to the muscle bath via a warming coil from a reservoir. Tyrode's solution in the muscle bath and in the reservoir is aerated with oxygen. The pH of the solution in the muscle bath is about 6.8. Some of the rat ileal strips used were stored for up to six hours at 5° C. in oxygenated Tyrode's solution.

Agents, the effects of which are to be studied, are dissolved in glucose-free Tyrode's solution and added to the bath in 0.5 ml. quantities from a 1 ml. tuberculin syringe, or are dissolved in reservoir fluid and admitted to the muscle bath through a side-arm instead of the usual drug-free solution.

The Tyrode's solution used in the experiments has the following composition in grams/liter:

Sodium Chloride	8.0
Potassium chloride	0.2
Calcium chloride dihydrate	0.2
Sodium bicarbonate	1.0
Sodium dihydrogen phosphate	0.05
Magnesium chloride hexahydrate	0.1
Glucose	1.0

Concentrations of drugs are expressed in grams per milliliter in exponential form. Drugs are prepared as stock solutions in glucose-free Tyrode's solution; stock solutions of acetylcholine (1 mg./ml., or 10^{-3}) are acidified to pH 2 with acetic acid. Solutions are prepared for use by dilution of stock solutions with glucose-free Tyrode's solution. Fresh stock solutions are prepared every three to five days and are refrigerated at 5° C. when not in use.

RESULTS:

The more than eighty experiments performed were done in groups which differed slightly from each other in experimental design. We shall summarize the work by describing the results obtained in each type of experiment. Since the rat ileum has hardly been used in biological assays, a large number of our experiments were performed in order to characterize the biological preparation.

A. In the first series of eleven experiments, concentrations of acetylcholine between 10^{-9} and 10^{-5} were used. The effect of each concentration was studied after exposure of the tissue to that concentration for periods from 0.25 to 2 minutes. After each exposure the tissue was washed three times at one minute intervals with drug-free Tyrode's solution; drug administrations were repeated at five minute intervals. Then a dose-effect curve for acetylcholine was determined, using three to five dose levels for each curve, and from five to twelve observations in each curve. Data for two such curves were determined similarly in each experiment; between the two determinations of the curves, the muscle was exposed to an acetylcholine concentration of 10^{-4}.

For concentrations of acetylcholine between 10^{-8} and 10^{-5}, inclusive the modal duration of exposure to produce maximum contraction of the muscle was 1 minute; in all subsequent experiments the tissue was exposed to

acetylcholine for 1 minute. For concentrations of acetylcholine between 10^{-8} and 10^{-4}, inclusive, the muscle relaxed completely, so that the tone was the same as that before drug administration, after one or two changes of bath fluid; in all subsequent experiments the tissue was washed three times at one minute intervals after each exposure to acetylcholine.

It was found that log dose-probit response curves for the system were essentially linear. In all the experiments and all subsequent experiments, responses of the muscle (measured in millimeters from the kymograph tracing) were expressed as a percentage of the maximum response of the muscle observed during the course of the experiment. The percentages were converted to probits and plotted against the logarithms of the corresponding acetylcholine concentrations; straight lines fitting the plotted data were fitted by eye.

The slopes of the two dose-effect curves determined in each experiment were computed, as were the median effective acetylcholine concentrations (EC50, determined by interpolation). The data were summarized using the non-parametric tests of Wilcoxon (Biometric Bull. 1:80, 1945) and Jackson and Ross (Am. Stat. Assoc. J. 50:416, 1955).

The mean EC50 for the curves determined before exposure to the high concentration of acetylcholine was 7.2×10^{-8} and ranged from 1.7×10^{-8} to 7×10^{-7}; the mean EC50 after exposure to the high concentration of acetylcholine was 6.1×10^{-7} and ranged from 5.6×10^{-8} to 5.4×10^{-5}. These means were statistically different ($P<0.01$). Analysis of the mean change in EC50 for the experiments indicated also that a significant ($P<0.05$) decrease in sensitivity of the muscles occurred after exposure to concentrations of acetylcholine of 10^{-4}. Observation of the effects of constant concentrations of acetylcholine (10^{-6}) repeated at intervals throughout the experiment indicated that no significant or consistent changes in responsiveness of the tissue occurred after exposure to concentrations of acetylcholine of 10^{-5} or less.

The mean slope of the dose-effect curves before exposure to the high concentration of acetylcholine was 0.782 and ranged from 0.50 to 1.20; the mean slope after exposure to the high acetylcholine concentration was 0.771 and ranged from 0.45 to 1.20. The mean slopes were insignificantly different at the 0.1 level. Paired comparison also revealed that no significant change in slope ($P>0.1$) occurred after exposure of the tissues to high concentrations of acetylcholine.

The results indicated that the rat ileum was reasonably sensitive to the spasmogenic effects of acetylcholine, and that the results from rat to rat were reasonably consistent. The system seemed appropriate to the development of a straight-line assay since even the loss of sensitivity (increased EC50) caused by high concentrations of acetylcholine was not acompanied by loss of responsiveness of the tissue and possible loss of parallelism of dose-effect curves involved in an assay.

B. In a series of four experiments an assay system for acetylcholine was designed and tested. Each experiment was composed of from eight to ten

twenty-minute periods. During each period, the tissue was exposed to four logarithmically spaced concentrations of acetylcholine (10^{-7} to 3×10^{-6}) in random order; two of the concentrations were designated as "known," two as "unknown." The data collected during each period were used to draw two two-point log dose-probit effect curves, one for the "known" and one for the "unknown"; the EC50's and slopes of the two curves were determined. The data were summarized statistically using the methods described above and by using the Spearman rank-difference correlation coefficient.

When the mean differences between the EC50's for the "known" samples ($EC50_k$) and the "unknown" samples ($EC50_u$) were determined, it was found that in none of the four experiments was there a significant mean difference in the EC50's ($P>0.05$); similarly, in three of the four experiments there was an insignificant mean difference ($P>0.05$) in the slopes of the dose-effect curves for the "known" and "unknown" samples. In any given period the two EC50's determined were independent estimates of the same theoretical EC50 and the two slopes determined were independent estimates of the same theoretical slope. In any given period the acetylcholine content of an "unknown" sample could be measured reliably.

When the $EC50_k$'s or $EC50_u$'s, respectively, were correlated with the time during the experiment at which they were determined, it was found that the correlation coefficient was of positive sign in seven of eight cases, but that the magnitude of the coefficient was significantly different from zero ($P<0.01$) in only three of eight cases. A similar analysis of the slopes of the dose effect curves showed that slope was negatively correlated with time in all cases but that the correlation was significant in only two of eight cases. Although the sensitivity of the preparations tended to decrease with time (increase of EC50) and the reactivity tended to decrease with time (decrease in slope), the changes were usually not significant.

When, for each experiment, $EC50_u$'s were correlated with $EC50_k$'s, it was found that in three of the four cases the degree of correlation was insignificant ($P>0.05$); similarly the correlation within each experiment between the slopes of the dose-effect curves for the "known" and "unknown" samples were insignificant ($P>0.05$) in three of four cases. During any experiment, although the preparations tended to lose sensitivity and reactivity with the passage of time, the changes in $EC50_u$ and $EC50_k$ varied essentially independently of each other; the same was true of the slopes of the curves determined for the "known" and "unknown" solutions.

In none of the experiments was there a significant (P equal to or less than 0.05) correlation between $EC50_u$'s and their corresponding slopes or between $EC50_k$'s and their corresponding slopes. In the course of an actual assay one would not anticipate a bias in estimates of acetylcholine concentration of the solution as a result of changes in slope of the dose-effect curve, at least when the degree of change was of the order of magnitude observed in these experiments.

A "4-point assay" of acetylcholine would be a reliable procedure, insignificantly influenced by "aging" of the preparation. Variability in

estimated acetylcholine concentration of a solution of unknown acetylcholine concentration was observed to occur randomly and could be minimized by replicate testing of the unknown solution.

C. A total of 32 experiments were performed to determine the effect of nicotine, hexamethonium, and neostigmine on the response of the rat ileum to acetylcholine. The experiments varied in design and in the sequences in which the various agents were administered. Because of the variability in design, uniform statistical treatment of the data was not possible, and we shall summarize the results of the experiments qualitatively.

(1) In a series of eight experiments in which the ileal strip was exposed for one minute periods alternately to varied concentrations of nicotine or acetylcholine, it was observed that nicotine in concentrations as high as 10^{-4} produced contractions of the ileum which were never more than 10% of the maximum contraction obtainable with acetylcholine. Concentrations of nicotine between 10^{-7} and 10^{-5}, inclusive, rarely produced contractions which were more than 2-3% of the maximum contraction height produced with acetylcholine. Concentrations of nicotine as high as 10^{-5} did not alter the response to subsequent administrations of acetylcholine; concentrations of nicotine of 10^{-4} decreased remarkably the sensitivity (increased the EC50) and reactivity (decreased the slope of the dose-effect curve) of the ileal strip to subsequent administrations of acetylcholine.

(2) In twenty-two experiments, dose-effect curves for acetylcholine were determined before, during, and after the rat ileal strip had been bathed in Tyrode's solution containing various concentrations of neostigmine (10^{-7} - 10^{-5}), nicotine (10^{-5}) and hexamethonium (10^{-5}), or combinations of these for periods of from twenty minutes to three hours. Concentrations of neostigmine of 10^{-7} left unchanged or decreased slightly the sensitivity of the preparation of acetylcholine; concentrations of neostigmine of 10^{-6} and 10^{-5} almost uniformly decreased the sensitivity to acetylcholine. When the sensitivity to acetylcholine was not altered by neostigmine, it was not altered by the subsequent addition of nicotine or hexamethonium to the Tyrode's solution.

D. A total of seventeen experiments have been performed using our assay system to measure acetylcholine release from the isolated guinea pig ileum, and to determine the effects of nicotine, hexamethonium and tetraethylammonium on this release.

After a guinea pig is killed, its entire ileum is removed and washed inside and out with magnesium-free Tyrode's solution. (Except for the absence of the magnesium chloride the Tyrode's solution has the composition given above.) The ileum is cut into 12-16 pieces of about equal size; a random pair of these segments are patted dry, weighed and placed into a small beaker containing 10 ml. of oxygenated magnesium-free Tyrode's solution containing neostigmine, 10^{-6}. Six such beakers are prepared. The contents of one beaker receives no further treatment. Two of the beakers are placed in boiling water for 10-20 minutes, and reconstituted to 10 ml. volume with oxygenated magnesium-free Tyrode's solution; one of these beakers

receives no further treatment; to one is added the highest concentration of nicotine, hexamethonium, or tetraethylammonium to be studied in the experiment. To each of two other beakers is added one of two concentrations of the agent (nicotine, hexamethonium, or tetraethylammonium) to be studied. A sixth beaker, like the first, contains only neostigmine. All of the beakers are kept at 37° C. for at least one hour. In the case of all beakers but the first described above, after reconstitution or introduction of the nicotine, hexamethonium, or tetraethylammonium, or introduction of neostigmine the incubation of the beaker at 37° is carried out for five minutes; then the fluid contents of the beaker are removed, and immediately replaced by 10 ml. of fluid of identical composition, and incubated for 1 hour. Oxygen is bubbled into each beaker during incubation.

After the one hour incubation period, 6 ml. of fluid are removed, diluted with 3.0 ml. of acetic acid solution of pH 3-4, diluted further with Tyrode's solution containing neostigmine (10^{-6}) and assayed against standard concentrations of acetylcholine, using a 4-point parallel-line assay using a random dose order. Equipotent quantities of the standard and unknown solutions are equvalent to about an EC20 (concentration required to produce a contraction height 20% of the maximum obtainable). Using the data, the acetylcholine output from the guinea pig ileal segments are calculated in units of micrograms of acetylcholine/gram of tissue wet weight/hour. All samples are assayed in duplicate; the two results are averaged. Comparisons between the means are made by means of the "G" test of Jackson and Ross.

After each sample has been assayed in duplicate, the rat ileum is exposed to atropine (10^{-5} - 10^{-4}), and both standards and the largest volume of each sample tested previously are "reassayed." In no case, in which the response of the ileum to the standard has been abolished, have unknown solutions produced shortening of the ileal strip.

RESULTS:

In two preliminary experiments it was observed that the acetylcholine output in the presence of neostigmine (10^{-6}) and after an incubation of one hour, was 0.625 and 0.602 micrograms/gram/hour, respectively. Under similar conditions, but in the presence of nicotine (10^{-5}), the acetylcholine outputs were 0.502 and 0.232 mcgms/gm/hr., respectively. When after five minutes of incubation in the presence of nicotine (10^{-5}) the fluid contents of the beakers was discarded and immediately replaced before incubation for one hour, the acetylcholine outputs for the one hour period were 0.183 and 0.000 mcgm/gm/hr., respectively. We interpret these results to mean that immediately after administration of nicotine to the ileal strip, rather large amounts of acetylcholine may be released, i.e. the "stimulant" phase of nicotine action occurs; this acetylcholine accumulates in the incubation medium and, if not removed, can mask the decreased amounts of acetylcholine released during the "paralytic" phase of nicotine action. In all subsequent experiments the effects of nicotine, hexamethonium, and tetraethylammonium on acetylcholine output were measured during the one hour period following the preliminary five minute incubation period.

In order to determine whether administration of neostigmine might have early effects on acetylcholine output, as might be anticipated from the work of Riker, et al. (J. Pharmacol. 121:280, 1957) we measured acetylcholine output from strips incubated for one hour in the presence of neostigmine alone and from strips, similarly treated with neostigmine, but incubated for one hour only after a preliminary five-minute incubation period. The data are given in the first two columns of the table. Analysis of these data indicate that the mean difference in mean acetylcholine output under the two conditions was insignificantly different from zero ($P>0.05$), i.e. neostigmine had no significant effect on acetylcholine during the first five minutes of incubation.

The results of the experiments are given in the table.

It can be seen that in the experiments using nicotine, the mean acetylcholine release in unboiled tissue treated with only neostigmine was 0.492 mgm/gm/hr. Solutions which had contained guinea pig tissue heated in a boiling water bath caused no contraction of the rat ileum. Such solutions still produced no response in the rat ileum even when they contained nicotine (10^{-5}). Nicotine (10^{-5}) completely prevented acetylcholine release in all five experiments. Nicotine (10^{-6}) completely prevented acetylcholine release in three experiments, and increasing the amount released in two. Since in these two experiments high concentrations of nicotine abolished acetylcholine release, we interpret the data as indicating that in these two strips nicotine (10^{-6}) was a "stimulating" rather than a "paralyzing" or "blocking" concentration. All of the decreases in acetylcholine output produced by nicotine, but neither of the increases, were significant at the 0.05 level of significance.

In the experiments with tetraethylammonium, the mean acetylcholine release in unboiled tissue was 0.499 mcgm/gm/hr. Slight amounts of acetylcholine were present in boiled tissue in two experiments. In every experiment tetraethylammonium ion (10^{-6} to 10^{-3}, inclusive) increased the amount of acetylcholine present in the incubation fluid, occasionally by several hundred per cent, but the increase was significant ($P<0.05$) in only three of ten cases. There is no clear relationship between tetraethylammonium concentration and amount or increase in acetylcholine concentration.

The mean acetylcholine release in experiments with hexamethonium was found to be 2.126 mcgm/gm/hr. for the unboiled tissue. The incubation fluid for boiled tissue was found to contain no acetylcholine; in one of three instances fluid for boiled tissue with added hexamethonium (10^{-4}) was found to contain acetylcholine. In all three experiments with hexamethonium (10^{-6} to 10^{-3} inclusive) the inclusion of hexamethonium in the incubation fluid resulted in a decreased measurable amount of acetylcholine, but the difference was significant ($P<0.05$) in only one of six cases. In two of three experiments there was less acetylcholine assayed in the incubation fluid containing the higher concentration of hexamethonium.

When the effectiveness of standard concentrations of acetylcholine were correlated with the time during each assay at which the respective concentrations were administered, it was found, in the case of experiments with nicotine

and hexamethonium, that there was, in general, a gradual but statistically insignificant ($P>0.1$) loss in sensitivity of the rat ileum preparation, i.e. decreased response to a standard concentration of acetylcholine. In experiments with tetraethylammonium, however, there was a progressive and significant ($P<0.05$) increase in effectiveness of standard concentrations of acetylcholine during the time course of the assays.

DISCUSSION:

We have previously put forward the hypothesis that impaired ganglionic transmission produced in the cat by administration of nicotine or tetraethylammonium is the result only of a decrease in acetylcholine release from pre-synaptic nerve terminals produced by the agents. On the other hand, our previous data were consistent with the hypothesis that hexamethonium impaired ganglionic transmission by preventing access of acetylcholine (released in normal amounts) to post-synaptic cell receptors.

The results reported above, obtained from experiments with nicotine, are consistent with our hypothesis: The amount of acetylcholine determined to be present in medium in which guinea pig ileal segments had been incubated was found to be decreased by nicotine. This was particularly true when care was taken to eliminate from the incubation fluid the acetylcholine released during the early "stimulant" phase of nicotine's action.

The results obtained with tetraethylammonium in these experiments are difficult to interpret, because the tetraethylammonium ion is known to have a number of actions which, exerted independently, but simultaneously, could yield the results we have observed. Under our conditions of incubation, and in the absence of detailed knowledge of processes in the guinea pig ileal wall, it is impossible to decipher the meaning of our results.

Tetraethylammonium ion, for example, has been shown to facilitate benzoylcholine hydrolysis by serum cholinesterase (Erdos, E., et al. Science 128:92, 1958); such an effect in our preparation would tend to decrease the amount of acetylcholine present in the incubation fluid. Kensler and Elsner, however, have shown that in the presence of low acetylcholine concentrations, tetraethylammonium decreases specific cholinesterase activity, but increases activity in the presence of high acetylcholine concentrations (J. Pharmacol. 102:196, 1951). This effect of tetraethylammonium was not observed by Kensler and Elsner when serum cholinesterase was used. Since we have no quantitative information on the kinds, concentrations, and kinetics of cholinesterases in the guinea pig ileum, little information of substrate concentration at the esteratic sites, and no information on the interaction of tetraethylammonium and neostigmine in the system, our results cannot easily be interpreted in terms of the effect of tetraethylammonium on the stability of acetylcholine after its release from nerve terminals. Tetraethylammonium might have other actions in the preparation which could account for our results. Stovner (Acta Pharmacol. et Toxicol. 15:55, 1958) and Koketsu (Am. J. Physiol. 193:213, 1958) have both presented evidence that tetraethylammonium can cause acetylcholine release at the skeletal neuromuscular

junction, and Cowan, et al. (J. Physiol. 91:101, 1937) have shown that tetraethylammonium can increase the spontaneous firing of at least isolated neurons. Such mechanisms as these could account for the results we have observed, could it be shown that the mechanisms were operative in the guinea pig ileum.

Paton and Zaimis (Brit. J. Pharmacol. 6:155, 1951) have reported that hexamethonium did not decrease acetylcholine output from the cat superior cervical ganglion at the time transmission was impaired by hexamethonium. However, Kensler and Elsner (ibid.) have reported that like tetraethylammonium, hexamethonium can increase or decrease specific cholinesterase activity, depending on acetylcholine concentration. We lack sufficient quantitative information to determine whether the phenomenon observed by Kensler and Elsner could account for our observation that less acetylcholine was present in incubation medium which contained hexamethonium than in medium which did not.

It should be pointed out that our assay procedure measures the acetylcholine content of incubation fluid; we can only infer that, in the case of nicotine, for example, total absence of acetylcholine in medium which contained low concentrations of nicotine is more likely to indicate impaired release of acetylcholine than only facilitations of its hydrolysis. Although the guinea pig ileum has proven to be a useful "acetylcholine generator" in the development of our assay procedure, it is not the present purpose of our work to study in detail the effects of agents on the guinea pig ileum, which contains several systems for release and degradation of acetylcholine. Hence, future work will follow two lines of endeavor:

(1) Assay of acetylcholine output from the cat superior-cervical ganglion. Our hypotheses of the mechanism by which nicotine, tetraethylammonium and hexamethonium impaired ganglionic transmission were derived from experiments using the relatively simple ganglionic transmission system. We shall use our assay procedure to measure acetylcholine content of the venous effluent from ganglia in which cholinesterase has been inactivated, during periods of normal transmission and when transmission has been impaired by the administration of nicotine, tetraethylammonium, and hexamethonium.

(2) The results obtained with tetraethylammonium and hexamethonium, particularly the change in sensitivity of the assay preparation after repeated exposure to tetraethylammonium, suggests that further investigations be made to determine the dependency of our results on the concentrations of tetraethylammonium and hexamethonium and the degree to which various concentrations of the agents influence the responsiveness of the assay strip.

Effects of Nicotine, Tetraethylammonium and Hexamethonium on Apparent Acetylcholine Output (in mcgms/gm/hr) from Isolated Guinea Pig Ileal Segments

Experiment	Drug	Conditions of Incubation*							
		Unboiled Tissue +	Unboiled Tissue	Boiled Tissue	Boiled Tissue Plus Drug	Unboiled Tissue Plus Drug			
						Drug 10^{-3}	Drug 10^{-4}	Drug 10^{-5}	Drug 10^{-6}
69	Nicotine	0.327	0.346	0.0	0.0	-	-	0.0 ‡	0.401
70		1.477	0.494	0.0	0.0	-	-	0.0 ‡	0.0 ‡
71		0.437	0.695	0.0	0.0	-	-	0.0 ‡	0.0 ‡
72		1.165	0.432	0.0	0.0	-	-	0.0 ‡	0.557
73		1.026	0.495	0.0	0.0	-	-	0.0 ‡	0.0 ‡
	Mean	0.887	0.492	0.0	0.0			0.0	0.194
77	Tetra-ethyl ammonium	1.105	0.342	0.0	0.0	-	0.612	0.575	-
78		0.554	0.458	0.0	0.0	-	0.792‡	0.844‡	-
79		0.207	0.257	0.018	0.0	-	0.657	0.268	-
80		2.062	0.710	0.0	0.0	1.589‡	1.063	-	-
81		0.864	0.726	0.0	0.747	-	-	0.887	1.261
	Mean	0.958	0.499	0.003	0.149	1.589	0.881	0.643	1.261
82	Hexa-methonium	0.740	3.081	0.0	0.0	-	-	1.055	0.614
83		1.138	0.715	0.0	0.314	-	0.0 ‡	0.610	-
84		0.890	2.583	0.0	0.0	0.915	1.207	-	-
	Mean	0.923	2.126	0.0	0.105	0.915	0.604	0.833	0.614

* All incubations were performed in the presence of neostigmine, 10^{-6}.

\+ Incubations performed for one hour; for other columns, incubations performed for one hour after preliminary five minute incubation (see text).

‡ Difference from corresponding control value significant at 0.05 level of significance.